Biosynthesis of a Hydroxy Fatty Acid Polymer, Cutin. Identification and Biosynthesis of 16-Oxo-9- or 10-hydroxypalmitic Acid, a Novel Compound in *Vicia faba*†

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ABSTRACT: In the cuticular polymer, cutin, of young *Vicia faba* leaves 16-oxo-9-hydroxypalmitic acid and its 10-hydroxy isomer were identified by combined gas–liquid chromatography–mass spectrometry of the products derived by $LiAlH_4$ and $LiAlD_4$ treatment of the polymer as well as hydroxylamine treated polymer. In the disubstituted C_{16} acid fraction the major portion of the 9-hydroxy acid contained an aldehyde function at the ω carbon while only a small portion of the 10-hydroxy isomer contained the 16-oxo function. As the leaves developed the 9-hydroxy isomer decreased from 50% in the youngest tissue to 10% in the mature leaf. These structural changes were also shown by incorporation of [1-^{14}C]-palmitic acid into the 9-hydroxy and 10-hydroxy isomers in the developing leaves as measured by the distribution of radioactivity among the CrO_3 oxidation products. These results for the first time clearly show that dramatic structural changes occur in cutin as a plant tissue develops. That [1-^{14}C]palmitic acid was incorporated into 16-oxo-9- or 10-hydroxypalmitic acid was shown by isolation and identification of the dimethyl acetal, semicarbazone, and oxime of the labeled aldehyde. Exogenous [G-^{3}H]-16-hydroxypalmitic acid was converted into 16-oxo-9- or 10-hydroxypalmitic acid in young *V. faba* leaves without involving degradation of the carbon chain. Direct conversion of 10,16-dihydroxypalmitic acid into 16-oxo-10-hydroxypalmitic acid was also demonstrated in this tissue.

Proteins and chitin constitute the major polymeric materials in the outer layer of animals. However, the structural component of plant cuticle is a polymer of hydroxy fatty acids called cutin (Martin and Juniper, 1970; Kolattukudy and Walton, 1972b). Recent structural studies showed that this biopolymer is made up of various combinations of a C_{16} family and a C_{18} family of hydroxy acids (Eglinton and Hunneman, 1968; Walton and Kolattukudy, 1972a; Croteau and Fagerson, 1972; Brieskorn and Kabelitz, 1971). Palmitic acid, ω-hydroxypalmitic acid, and 10,16- or 9,16-dihydroxypalmitic acid or its other positional isomers are the major components of the former, while oleic acid, ω-hydroxyoleic acid, 18-hydroxy-9,10-epoxystearic acid, 9,10,18-trihydroxystearic acid, and their Δ^{12}-unsaturated analogs make up the bulk of the latter family. More recently it was noted that the chemical composition of the cuticular polymer may change with the age of the tissue; in very young tissues several novel compounds were discovered (Kolattukudy, 1972, 1973; Kolattukudy *et al.*, 1973). In a preliminary communication it was noted that in the cutin of the embryonic shoots of *Vicia faba* 16-oxo-9-hydroxypalmitic acid is a major component (Kolattukudy, 1972). However, nothing is known about the biosynthesis of these compounds. In this paper the identification and biosynthesis of this novel compound are described. It is also shown that synthesis of 16-oxo-9- or 10-hydroxypalmitic acid occurs mainly during the early phase of cutin synthesis in the very young tissue.

Experimental Section

Plants. Broad bean (*V. faba*) plants were grown as described earlier (Kolattukudy and Walton, 1972a) and leaves from plants of the 6–9 leaf stage were used. The leaf age is expressed in terms of the radius of the leaf. The numbers used represent an average range. Since there are variations in the rate of growth of leaves from batch to batch, comparisons are made only among leaves picked from a given batch of plants. Leaves under 5 mm in radius were used intact while larger leaves were sliced into strips of about 5 mm with a razor blade. In experiments where apical bud was used they were excised under a magnifying glass with a razor blade. All tissues were thoroughly washed with water and blotted with filter paper before incubation with substrates.

Substrates and Reagents. [1-^{14}C]Palmitic acid (specific activity 55 Ci/mol) was purchased from Amersham/Searle. [G-^{3}H]-10,16-Dihydroxymethyl palmitate (2850 Ci/mol) was prepared in the following manner. *V. faba* cutin isolated as described before (Kolattukudy and Walton, 1972a) was depolymerized by refluxing it with 14% BF_3 in CH_3OH for 24–48 hr. 10,16-Dihydroxymethyl palmitate was isolated by repeated thin-layer chromatography (tlc) with ethyl ether–hexane–methanol (20:5:1 v/v). After checking the purity by gas–liquid chromatography (glc) of the trimethylsilyl ether and mass spectrometry (the sample contained about 10% 9,16-dihydroxymethyl palmitate which could not be removed) about 25 mg of the material was exposed to 6Ci $^3H_2$ at New England Nuclear Corp., Boston, Mass., according to the Wilzbach's method. Rigorous purification of the methyl ester by repeated tlc followed by hydrolysis and purification of the acid by tlc with ethyl ether–hexane–methanol–formic acid (40:10:1:2) gave chemically and radiochemically pure [G-^{3}H]-10,16-dihydroxypalmitic acid (containing 10% of the 9-hydroxy isomer). [G-^{3}H]-ω-Hydroxypalmitic acid (440 Ci/mol) was prepared in a similar manner. $LiAlD_4$ (minimum isotopic purity 99 atom % D) was purchased from Merck, Sharp and Dohme of Canada. The cutinase was isolated from the culture fluid of *Fusarium solani Pisi* as described before (Purdy and Kolattukudy, 1973). Other reagents and solvents

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were obtained from the usual commercial sources. Substrate dispersions were prepared with the aid of Tween 20 as described before (Kolattukudy and Walton, 1972a).

Incorporation of [1-^{14}C]Palmitic Acid into Crude Cutin Preparation. Generally 1–2 g of leaves or leaf slices were mixed thoroughly with 0.5–1 ml of substrate solution in 125-ml erlenmeyer flasks and they were incubated in a shaking (100 rpm) water bath at 30° for 3 or 4 hr. (Specific information is given under the tables.) At the end of the experimental period the leaf slices were ground in a Ten Broek homogenizer with about 15 ml of water. The homogenate was centrifuged at 27,000*g* for 15 min, the insoluble material was collected, and the soluble lipids were throughly extracted from it as described before (Kolattukudy and Walton, 1972a). The solvent used in the final wash was the one used in the subsequent treatment. Although final residue contained many polymeric materials other than cutin, all the ^{14}C derived from [1-^{14}C]palmitic acid contained in this residue was in cutin (Kolattukudy and Walton, 1972a).

Determination of the Changes in Isomer Composition in the Dihydroxypalmitic Acid with Age of the Leaves. Leaves of appropriate age, selected as described in a previous section, were homogenized and the crude cutin preparation was obtained from them as described under incorporation experiments. The crude cutin was treated with either $LiAlH_4$ or $LiAlD_4$ and the products were isolated as described before (Kolattukudy and Walton, 1972a). The thin-layer chromatographically pure C_{16}-triol fractions were subjected to glc–ms as described before as their trimethylsilyl ethers, and the spectra were recorded at the apex of the gas chromatographic peak. Where changes in biosynthetic patterns were examined, the tissues of various ages were incubated with [1-^{14}C]palmitic acid (10 μCi, 180 nmol) for 4 hr at 30°. The isolation and hydrogenolysis of crude cutin as well as isolation of products were done as described elsewhere in this paper. The labeled C_{16}-triol fraction purified by thin-layer chromatography (ethyl ether–hexane–methanol (8:2:1 v/v)) was subjected to CrO_3 oxidation followed by product analysis as described before (Kolattukudy and Walton, 1972a).

Identification of Labeled 16-Oxo-9- or 10-Hydroxypalmitic Acid by Means of Its Acetal Derivative. The crude cutin preparation obtained from young (2–4 mm) *V. faba* leaves which had been incubated with [1-^{14}C]palmitic acid for 4 hr was refluxed with 14% BF_3 in methanol for 48 hr. After addition of 2 volumes of water the products were extracted three times with chloroform. The chloroform-soluble material was subjected to tlc on 1-mm layers of silica gel G with ethyl ether–hexane (7:3 v/v) as the solvent and with ω-hydroxymethyl palmitate and 10,16-dihydroxymethyl palmitate as standards. The labeled components were detected with a Berthold thin-layer radioactivity monitor.

The labeled fraction that migrated between the ω-hydroxymethyl ester fraction and 10,16-dihydroxymethyl palmitate was recovered. This fraction, tentatively designated as acetal fraction, was refluxed with 15 ml of a 1:14 (v/v) mixture of concentrated H_2SO_4 and water containing 0.5% dinitrophenylhydrazine for 5 hr under N_2. The reaction mixture was extracted four times with chloroform. When an aliquot of the recovered products was subjected to tlc with ethyl ether–hexane–methanol–formic acid (40:10:1:2) two labeled components were found. Since they were suspected to be free acid and methyl esters, the material was refluxed with 14% BF_3 in CH_3OH for 2 hr. The products isolated from this reaction mixture showed only one component when subjected to tlc in the above solvent system. The 2,4-dinitrophenylhydrazone was recovered from the silica gel with methanol and it was refluxed with 20 ml of a 10:1 mixture of dioxane and water containing 1 g of pyruvic acid for 2 hr under N_2. The products were extracted three times with chloroform and evaporated to dryness, and then treated with $NaBH_4$ in dioxane, at room temperature for 15–30 min. The products recovered in the usual manner were subjected to tlc with 10,16-dihydroxymethyl palmitate as standard and ethyl ether–hexane–methanol–formic acid (40:10:1:2 v/v) as the solvent. Occasionally the aldehyde generated by the pyruvic acid treatment was also isolated and chromatographed before subjecting it to $NaBH_4$ treatment. The generation of aldehyde from the hydrazone gave only 20–40% yield and therefore the unreacted hydrazone was isolated and the pyruvic acid treatment and reduction was repeated and the pooled dihydroxymethyl palmitate was acetylated. Acetylated products were purified by tlc and then subjected to radio gas–liquid chromatography.

Analysis of the labeled material released by BF_3–CH_3OH treatment (24 hr) showed that a considerable amount (20–40%) of ^{14}C remained in the origin in ether–hexane (70:30 v/v) solvent system. This material did not give rise to significant amounts of the recognized monomer derivatives either with further BF_3–CH_3OH treatment or with $LiAlH_4$ in tetrahydrofuran. Such a polar material was not found when crude cutin was subjected to alkaline hydrolysis followed by $LiAlH_4$ treatment (Kolattukudy and Walton, 1972a). Therefore, the polar material obtained from the BF_3–CH_3OH treatment of the crude cutin preparation is considered to be products (probably acetal type) of the reaction between labeled hydroxy acids and the unlabeled carbohydrates and/or other material contained in the crude preparation. Therefore, this technique could be used only for identification purposes but not for quantitation.

Identification of Labeled 16-Oxo-9- or 10-Hydroxypalmitic Acid by Means of Semicarbazone Derivative. The crude cutin preparation (from 2 g of tissue) was treated with pectinase and cellulase as described before (Walton and Kolattukudy, 1972a,b). The insoluble material was collected by centrifugation. This residue was washed twice with water and then it was suspended in 15 ml of water containing 1 g of semicarbazide and 1 g of sodium acetate. The reaction mixture was refluxed for 3 hr under nitrogen. The insoluble material was collected by centrifugation at 15,000*g* for 15 min. The pellet was washed two times with water and then with 0.2 M phosphate buffer (pH 8.0). The insoluble material was treated with 4 mg of cutinase (Purdy and Kolattukudy, 1973) in 4 ml of 0.2 M phosphate buffer (pH 8.0) under N_2 for 6 hr. The reaction mixture was centrifuged at 15,000*g* for 15 min and the pellet was suspended in 20 ml of water and centrifuged again. The combined supernatant was acidified and extracted three times with chloroform. The chloroform-extracted products were then subjected to tlc with ethyl ether–hexane–methanol–formic acid (40:10:1:2 v/v) as the solvent. A labeled component that migrated below 10,16-dihydroxypalmitic acid was recovered from the silica gel with methanol. This component, which could not be completely eluted (~80%), was tentatively designated the semicarbazone fraction. This fraction was treated with pyruvic acid and the product was reduced with $NaBH_4$ as described in the previous section. The final product was subjected to tlc in ethyl ether–hexane–methanol–formic acid (40:10:1:2) with 10,16-dihydroxypalmitic acid as a standard. The labeled component which corresponded to the standard was recovered. A portion of this material was

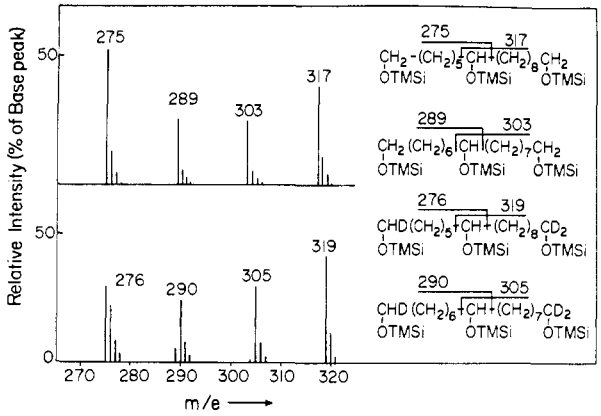


FIGURE 1: Partial mass spectra of the Me_3Si ethers of C_{16} triol derived by hydrogenolysis (top) and deuterolysis (bottom) of cutin from young (4 mm) leaves of *V. faba*. Spectra were recorded with 70-eV ionizing voltage at the apex of the gas chromatographic peak.

subjected to chromic acid oxidation and another portion to methylation and acetylation.

Identification of Labeled 16-Oxo-9- or 10-Hydroxypalmitic Acid by Means of Its Oxime. The crude cutin derived from 2.4 g of leaves (*ca.* 4 mm) was suspended in 10 ml of ethanol and 4 ml of pyridine containing 2 g of hydroxylamine hydrochloride. The reaction mixture was refluxed for 2 hr under N_2 and then centrifuged at 15,000*g* for 15 min. The residue was washed once with ethanol and twice with tetrahydrofuran. The washed residue was refluxed for 48 hr with $LiAlH_4$ in tetrahydrofuran. After decomposing the excess $LiAlH_4$ with water the mixture was acidified and extracted four times with chloroform to obtain a neutral fraction. The aqueous phase was made basic with KOH and then extracted four times with chloroform to obtain a basic fraction. After assaying aliquots for ^{14}C, both fractions were subjected to tlc.

Experiments with Embryonic V. faba Tissue. V. faba seeds (Burpee Co.) were surface sterilized by immersing them in 25% Chlorox solution for about 5 min. The seeds were washed thoroughly and placed on moist paper and germinated at room temperature for 5 days. The embryo was removed and the shoot and radicle portions were separated with a razor blade, and the tissue was thoroughly washed in deionized distilled water. A weighed amount of the tissue was well mixed with 0.5–1 ml of [1-^{14}C]palmitic acid (91 nmol, 11 $\times$ 10^6 dpm) solution and incubated at 30° for 4 hr. At the end of the incubation tissue slices were homogenized in water and the insoluble material was processed in the same manner as the leaf tissue.

Preparation of Other Derivatives. O-Acetylation was done overnight at room temperature with a 2:1 mixture of acetic anhydride and pyridine. N-Acetylation was done at room temperature with 1 ml of acetic anhydride in 5 ml of methanol (Gaver and Sweeley, 1966) and the products were purified by tlc. The N-acetylation was not complete under this condition. Therefore, the unreacted material was recovered and reacetylated to obtain an additional amount of N-acetylated material. Chromic acid oxidation was done as described previously and the products were purified by tlc before glc (Kolattukudy and Walton, 1972a). Trimethylsilylation was done by heating the sample with *N,O*-bis(trimethylsilyl)acetamide (Pierce Chemicals) for 15 min at 90°. Methyl esters were prepared by refluxing the acids with 14% BF_3 in CH_3OH.

Chromatography. Thin-layer chromatography was done with activated 0.5–1-mm layers of silica gel G in lined tanks. The solvents used are indicated in appropriate places in the text. Radio gas–liquid chromatography was done under conditions which are described in the legends to the figures and tables with a Perkin-Elmer 800 gas chromatograph attached to a Barber Colman radioactivity monitor. For structure determination a combination of a Varian Aerograph Model 328 gas chromatograph with a Perkin-Elmer-Hitachi mass spectrometer RMU6D was used with a Bieman separator interphase. Other details are given under the figures.

Determination of Radioactivity. Radioactivity in lipid samples and thin-layer chromatographic fractions was determined with a Packard liquid scintillation spectrometer (Kolattukudy, 1965). Internal standards were always used to determine efficiency (usually 70%). All counting was done with a standard deviation less than 3%. The ^{14}C in the effluent of the gas chromatograph was measured as described before (Kolattukudy, 1966).

Results and Discussion

Recent structural studies on the cutin from embryonic *V. faba* (Kolattukudy, 1972) and very young apple fruits (Kolattukudy, 1973; Kolattukudy *et al.*, 1973) suggested that the composition of this polymer may depend on the developmental stage of the tissue. In order to investigate this possibility, hydrogenolysis products and deuterolysis products of cutin from very young leaves (3–4 mm in diameter) were examined by tlc and combined glc–ms. Thin-layer chromatograms showed three components, alkanetriol, alkanediol, and alkan-1-ol. Gas–liquid chromatography of the triol fraction showed that it contained only C_{16} triol. The mass spectrum of the triol obtained by hydrogenolysis showed a weak molecular ion at 490, and fragment ions at *m/e* 475 ($M^+ - CH_3$), 400 ($M^+ - (CH_3)_3SiOH$), and 385 ($M^+ - CH_3 - (CH_3)_3SiOH$) confirming that it was a saturated C_{16} triol. Four strong ions were observed at *m/e* 275, 289, 303, and 317, strongly suggesting that the triol component was a mixture of 1,7,16-trihydroxyhexadecane and 1,8,16-trihydroxyhexadecane. The percentage of the two isomers can be calculated on the basis of the relative intensities of the α-cleavage ions as follows: % 8 isomer = (289 + 303)/(275 + 289 + 303 + 317). Such a calculation showed that about 35% of the triol was the 8 isomer. Similar calculations from the spectra obtained with cutin from mature *V. faba* leaves (Kolattukudy and Walton, 1972a) showed that only about 10% of the C_{16} triol was the 8 isomer suggesting that developmental stage affects cutin composition.

The mass spectrum of the triol obtained by $LiAlD_4$ treatment of the cutin from the young leaves showed ions at *m/e* 493 (M^+), 478 ($M^+ - CH_3$), 403 ($M^+ - (CH_3)_3SiOH$), and 388 ($M^+ - CH_3 - (CH_3)_3SiOH$) in addition to the ions at 492, 477, 402, and 387. These ions indicated that the triol sample contained dideuterated and trideuterated chains. The relative intensities of ions at 103, 104, and 105 [$CH_2OSi(CH_3)_3$, $CHDOSi(CH_3)_3$, and $CD_2OSi(CH_3)_3$] indicated that the terminal carbon carried 0, 1, and 2 deuterium atoms, respectively. These results suggested that a portion of the triol originated from 16-oxo-9- or 10-hydroxyhexadecanoic acid. Confirming this conclusion, the major α-cleavage ions from the deuterated triol were observed at *m/e* 275, 276, 290, 305, and 319 (Figure 1).

Ions at 276 (corrected for the isotope contribution from 275) and 290 represent the α-cleavage fragments originating from the aldehyde containing end of 10-hydroxy and 9-hydroxy derivative, respectively, whereas those at 275 and 289

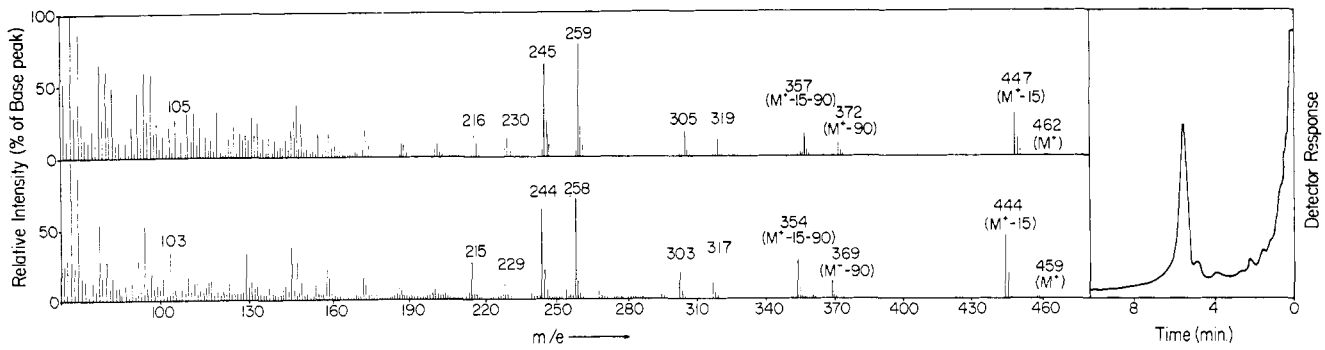


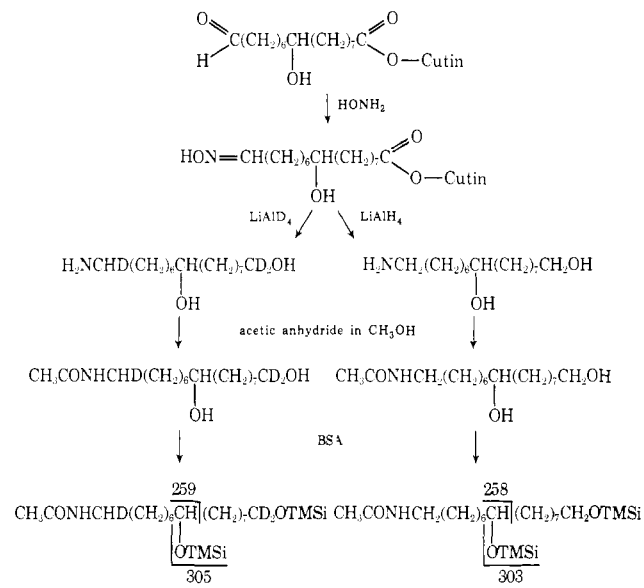
FIGURE 2: Gas–liquid chromatogram and the mass spectra of the Me_3Si ethers of the N-acetylated amino alkanediols obtained from $LiAlH_4$ treatment (bottom) and $LiAlD_4$ treatment (top) of hydroxylamine-treated cutin of young leaves of *V. faba*. The spectrum was recorded at the apex of the major peak with 70-eV ionizing voltage. Glc was done with 5 ft × 1/8 in. coiled stainless steel column packed with 5% SE-30 on 60–80 mesh Chromosorb W held at 260° with 23 psi inlet pressure.

represent the corresponding ω-hydroxy derivatives. Therefore 276/(275 + 276) and 290/(289 + 290) give the percentages of the aldehyde components in the 10-hydroxy acid and 9-hydroxy acid, respectively. Such a calculation showed that in the triol derived from the cutin of young *V. faba* leaves (2–4 mm) a small amount (about 30%) of the 10-hydroxy isomer originated from the 16-oxo compound while a major portion (about 80%) of the 9-hydroxy isomer resulted from the corresponding 16-oxo compound.

In order to confirm the occurrence of the 16-oxo-9- or 10-hydroxypalmitic acid in cutin, the insoluble residue obtained from very young *V. faba* leaves by thorough extraction with water and organic solvents was treated with hydroxylamine hydrochloride. The polymer, presumably containing the oximes, was depolymerized by exhaustive hydrogenolysis with $LiAlH_4$ which simultaneously should have reduced the oximes to amines. The basic fraction isolated from such a reaction mixture when subjected to thin-layer chromatography ($CHCl_3$–CH_3OH–NH_4OH 65:15:4 v/v) revealed one major component with an R_F of 0.28. This component was subjected to N-acetylation followed by trimethylsilylation of the hydroxyl groups. This derivative when subjected to gas–liquid chromatography showed one major component (Figure 2). The mass spectrum of this component showed a weak molecular ion at *m/e* 459 and a strong fragment ion at *m/e* 444 ($M^+ - CH_3$). Fairly intense ions were observed at *m/e* 369 ($M^+ - (CH_3)_3SiOH$) and 354 ($M^+ - CH_3 - (CH_3)_3SiOH$). These are the major ions expected from *N*-acetyldi(trimethylsiloxy)hexadecylamine.

The presence of a fairly strong ion at *m/e* 103 ($CH_2OSi(CH_3)_3$) showed that one of the trimethylsiloxy functions was present on a terminal carbon. The position of the other trimethylsiloxy function could be determined by the α-cleavage ions. One very strong pair of α-cleavage ions at *m/e* 244 and 258 obviously originated from the amino end of the molecule. The other pair of moderately strong α-cleavage ions originating from the opposite side of the molecule were found at *m/e* 303 and 317. These ions show that the glc peak constituted a mixture of *N*-acetyl-7,16- and 8,16-ditrimethylsiloxyhexadecylamine. If these structures originated as shown in Scheme I, use of $LiAlD_4$ instead of hydride in the reduction step should give rise to their trideuterio analogs. In such analogs two deuterium atoms should be located in the carbon carrying the primary alcohol function and one deuterium on the carbon carrying the amino group. The mass spectrum of the product obtained with $LiAlD_4$ in fact showed a molecular ion at *m/e* 462 and fragment ions at 447 ($M^+ - CH_3$), 372 ($M^+ - (CH_3)_3SiOH$), and 357 ($M^+ - CH_3 - (CH_3)_3SiOH$) showing

SCHEME I



that three deuterium atoms were incorporated. The fact that the nitrogen containing α-cleavage ions were found at *m/e* 245 and 259 showed that one deuterium atom was incorporated into this part of the molecule. α-Cleavage ions representing the other side of the molecules were found at *m/e* 305 and 319 (2 amu higher than the corresponding ions obtained with $LiAlH_4$) showing that two deuterium atoms were incorporated into that side of the molecule (Figure 2). The occurrence of a fairly strong ion at *m/e* 105 showed that two deuterium atoms were located on a terminal carbon. These results show that the basic component was 16-aminohexadecane-1,9- or -10-diol. Therefore these results show that cutin in very young leaves of *V. faba* contained 16-oxo-9 or 10-hydroxyhexadecanoic acid.

The semicarbazone derivative of the aldehydes was also prepared and the products were analyzed after reduction with $LiAlH_4$ and $LiAlD_4$ followed by trimethylsilylation. The conclusion regarding structures obtained from such studies was identical with that discussed with the oxime derivative.

From the deuterium labeling experiments already discussed it was determined that 62% of the 16-oxohydroxyhexadecanoic acid fraction contained the in-chain hydroxyl group at C-9. From relative intensities of the α-cleavage ions in the mass spectrum of the N-acetylated derivative discussed above (Figure 2) a value of 61% was obtained for the C-9 isomer. Similarly the measurement of the relative intensities

of the α-cleavage ions obtained from the semicarbazone derivative gave a value of 60% for the C-9 isomer. These data clearly agree in showing that the cuticular biopolymer in very young leaves (3 mm) of *V. faba* contains 16-oxo-9-hydroxyhexadecanoic acid and 16-oxo-10-hydroxyhexadecanoic acid in a ratio of approximately 3:2.

The occurrence of substantial amounts of the 9-hydroxy isomer and the presence of an aldehyde function at the ω carbon discussed above are at variance with the results of previous structural studies on cutin from mature leaves (Kolattukudy and Walton, 1972a). Therefore the two new structural features observed in the very young leaves may be attributed to the developmental stage. In order to test this possibility the content of the 9-hydroxy isomer in the cutin of *V. faba* leaves of different ages was determined by mass spectrometric technique. The triols isolated from the hydrogenolysates were subjected to glc–ms and the content of the 9,16-dihydroxypalmitic acid was determined from the relative intensities of the α-cleavage ions using the following formula: % 9-hydroxy isomer = (289 + 303)/(289 + 303 + 275 + 317), where each number represents the intensity of the ion at that *m/e* value. Results summarized in Table I show that as the leaves expanded the 9-hydroxy isomer decreased from 36 to 8–10%.

The aldehyde content of each isomer was determined by a technique involving deuterium labeling and mass spectrometry as described earlier in this section (Table II). In the apical bud more than 50% of the C_{16} triol originated from the 9-hydroxy isomer and as the leaves developed to full maturity only less than 10% was the 9 isomer. In the 9-isomer fraction of the in-chain hydroxylated C_{16} acids a major portion contained aldehyde function at the ω carbon at all stages of development.

TABLE I: Developmental Changes in the Distribution of Positional Isomers of 9- or 10,16-Dihydroxypalmitic Acid in *V. faba* Cutin.[a]

Half-width of the Leaf (mm)		% 9 Isomer	
Expt 1	Expt 2	Expt 1	Expt 2
	1–2		33.0
2		31.0	
	4–5		35.0
5		36.0	
	8		29.0
11		24.0	
	12		14.0
15		13.0	
	15		8.0
	18		9.0

[a] In each experiment, the C_{16} triol, isolated by tlc from the hydrogenolysate of crude cutin obtained from leaves of different ages, was analyzed by glc–ms with 5% SE-30 on 60–80 mesh Chromosorb W packed in 5 ft × 1/8 in. coiled stainless steel column held at 225°. The % 9 isomer was calculated from the relative intensities of the α-cleavage ions as described under the Experimental Section. Such determinations were done five times and results from two representative experiments are given here. Since different batches of plants with possible differences in growth rate were used averages were not taken. Generally, 18 mm represented mature or almost mature leaves.

TABLE II: Developmental Changes in the Composition of Disubstituted Hexadecanoic Acid in *V. faba* Cutin.

Age (Half-width) (mm)	Positional Isomer[c] %			
	9-Hydroxy		10-Hydroxy	
	16-Oxo	16-OH	16-Oxo	16-OH
0[a]	40.9	11.1	13.9	34.1
2–3	31.4	8.6	19.3	40.7
6–7	25.8	6.7	19.9	47.7
11–13	13.2	5.7	22.0	59.2
18[b]	6.1	2.8	12.1	79.8

[a] The apical buds 1 mm in diameter were excised with the use of a magnifying glass. [b] Mature leaves were used. [c] Aldehyde of 10 isomer = [276 − (275 × 0.3)]/[275 + (276 − 275 × 0.3)]; aldehyde of 9 isomer = [290 − (289 × 0.3)]/[289 + (290 − 289 × 0.3)]; total 9 isomer = [289 + (290 − 289 × 0.3) + 303]/[275 + (276 − 275 × 0.3) + 289 + (290 − 289 × 0.3) + 305 + 319]; where 275, 276, 289, 290, 305, and 319 represent intensities of ions with *m/e* values represented by these numbers. The factor 0.3 used for isotope correction was obtained by measuring the ratio of 276/275 and 290/289 in ten spectra of C_{16} triols obtained from hydrogenolysate of cutin samples. A 72-hr treatment of cutin with $LiAlD_4$ was used and the C_{16} triols were purified by tlc. The analysis was done as in Table I.

The proportion of the 10 isomer increased with age of the tissue and only a small portion of this isomer contained aldehyde function at the ω carbon. Thus as the leaves developed from the apical bud into the fully mature stage the proportions of the aldehyde-containing monomers and the 9 isomer in the in-chain substituted C_{16} acid fraction decreased dramatically. These results for the first time clearly show that dramatic structural changes occur in cutin as a plant tissue develops. The functional significance of such changes remains to be elucidated.

Biosynthesis of Cutin Acids in Embryonic V. faba. 16-Oxo-9-hydroxypalmitic acid was shown to be the major component of the cutin in embryonic *V. faba* by a deuterium labeling technique (Kolattukudy, 1972). In an attempt to study the biosynthesis of cutin in the embryonic tissue, it was found that ^{14}C was incorporated from [1-^{14}C]palmitic acid into the insoluble material only in the embryonic shoot and not in the radicle. For example, when 1.9 g of tissue was incubated with 91 nmol (11×10^6 dpm) of [1-^{14}C]palmitic acid for 3.75 hr at 30° the shoot incorporated 1.24×10^6 dpm into insoluble material while the radicle incorporated only about 0.05×10^6 dpm into the insoluble residue. Hydrogenolysis of the labeled insoluble material showed that all the ^{14}C was contained in ether-soluble products. Thin-layer chromatographic analysis of this product showed that 78, 12, and 10% of the label was in C_{16} triol, alkanediol, and alkanol fractions, respectively. These results clearly show that cutin synthesis occurs specifically in the portion of the embryo which is destined to become the aerial portion of the plant. Furthermore, the composition of the polymer in this very young tissue is similar to that of the mature leaves in that the bulk of the polymer is made of the C_{16} family of acids. More recently the flowers of *V. faba* were also found to have similar composition (P. E. Kolattukudy and R. Croteau, unpublished results). Thus, all

the aerial parts of a given plant may be covered with a very similar polymer.

In order to determine the position of the in-chain hydroxyl group in the labeled dihydroxypalmitic acid, the triols derived from it were subjected to chromic acid oxidation followed by radio gas–liquid chromatography. The bulk of the radioactivity of the products was contained in C_9, C_8, and C_{10} dicarboxylic acids in the order of decreasing amounts (Figure 3). Since the precursor was carboxyl-labeled palmitic acid the ^{14}C distribution among the CrO_3 degradation products shows that the C_{16} triol was derived from 9,16-dihydroxypalmitic acid and 10,16-dihydroxypalmitic acid. Labeled C_{16} triol was also detected by thin-layer chromatography in the hydrogenolysates of the soluble lipids, and chromic acid oxidation of this triol gave products identical with those obtained with the triol isolated from the insoluble material. Assuming that the C_{10} acid and an equal amount of C_9 acid originated from the 9-hydroxy isomer it was calculated that about 72% of the total triol originated from the 9-hydroxy isomer, and this value is in good agreement with the deuterium-labeling data which gave a value of 70% (Kolattukudy, 1972). In contrast, similar degradation of triol derived from [1-^{14}C]palmitic acid in *V. faba* leaves gave C_9 and C_{10} as the major products (Kolattukudy and Walton, 1972b). Thus, the major product derived from palmitic acid in the embryonic shoot was the 9-hydroxylated isomer while the 10-hydroxylated compound predominated in leaves.

The structural studies discussed in a previous section showed that the monomer structures in *V. faba* leaves depended on the developmental stage of the tissue. Therefore, biosynthetic studies were conducted with leaves of different ages in an attempt to demonstrate changes in biosynthetic patterns. The extent of incorporation of [1-^{14}C]palmitic acid into cutin changed with age (Table III) and these changes

TABLE III: Developmental Changes in Incorporation of [1-^{14}C]-Palmitic Acid into the Positional Isomers of 9- or 10,16-Dihydroxypalmitic Acid in *V. faba* Cutin.[a]

Leaf Size Half-width (mm)	Radioactivity in Triol (cpm × 10^{-6})	Distribution of ^{14}C among the Dicarboxylic Acids Derived by CrO_3 Oxidation of the Triols (%)		
		C_8	C_9	C_{10}
1.5	0.78	29.1	49.5	21.4
4.5	1.09	29.8	54.8	15.4
8.5	1.68	17.0	40.5	42.3
12	2.70	10.8	50.2	39.0
15	3.40	10.8	45.5	43.7
18	0.96	7.8	44.6	47.7

[a] In each case 1 g of tissue was incubated with 182 nmol (10 μCi) of [1-^{14}C]palmitic acid in a total volume of 1.0 ml for 4 hr at 30°. The crude cutin prepared was treated with $LiAlH_4$ for 24 hr and the products were analyzed by tlc. Each sample of labeled C_{16} triol was mixed with 1 mg of unlabeled C_{16} triol and oxidized with CrO_3 at 70–75° for 75 min, and the labeled dicarboxylic acids were esterified and purified by tlc (R_F of labeled dimethyl ester = 0.27 in hexane–ethyl ether–formic acid 65:35:2 v/v). The dimethyl esters were analyzed by radio glc with 5% SE-30 on 80–100 mesh Gas Chrom Q packed in 6 ft × 0.25 in. coiled stainless steel column held at 140°.

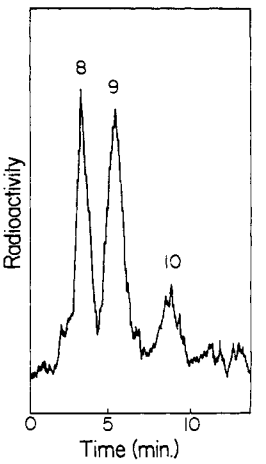


FIGURE 3: Radio gas–liquid chromatogram of the dicarboxylic acids (as dimethyl esters) derived by CrO_3 oxidation of the C_{16} triol isolated from the hydrogenolysate of the cutin derived from [1-^{14}C]palmitic acid in embryonic shoots of germinating *V. faba* seeds; 1.9-g tissue slices were incubated with 91 nmol (5 μCi) of [1-^{14}C]palmitic acid for 3.75 hr at 30°. Isolation and CrO_3 oxidation of products were done as described in the text. Glc was done with 4% OV-1 on 80–100 mesh Gas Chrom Q packed in 6 ft × 0.25 in. coiled stainless steel column held at 140° with 24 psi inlet pressure of argon. Identification of the components was done by comparing the retention times with those of authentic standards. The number on each peak represents the chain length.

were similar to those observed previously (Kolattukudy, 1970). Chromic acid oxidation of the C_{16} triol derived from [1-^{14}C]palmitic acid followed by radio gas chromatographic analysis of the products gave the results shown in Table III. Radioactivity in C_8 and C_9 represents the 9-hydroxy isomer while the radioactivity in C_9 and C_{10} originates from the 10-hydroxy isomer. It is clear that the synthesis of the 9 isomer decreased in relationship to the 10 isomer as the leaves developed. The patterns obtained with nearly mature leaves are identical with that previously reported (Kolattukudy and Walton, 1972b). These results together with the results of the structural studies discussed in a previous section clearly show that biosynthetic patterns of the monomers change with developmental stages of the tissue. The physiological significance of the change in the position of the in-chain hydroxyl group is not understood at the present time.

Incorporation of [1-^{14}C]Palmitic Acid into 16-Oxo-9- or 10-Hydroxypalmitic Acid. With the hydrogenolysis technique used in the biosynthetic experiments discussed above, 16-oxo-9- or 10-hydroxypalmitic acid and 9- or 10,16-dihydroxypalmitic acid gave indistinguishable triols. Therefore, with such techniques it was not possible to study the biosynthesis of the oxo compound. Therefore, the following methods were used to test whether palmitic acid was incorporated into 16-oxo-9- or 10-hydroxypalmitic acid.

(a) *Identification of the Labeled Acetal Derivative (Scheme II).* Insoluble material obtained from very young (2–4 mm) leaf slices incubated with [1-^{14}C]palmitic acid when treated with 14% BF_3 in methanol gave fairly complete depolymerization of the labeled cutin. When the soluble monomer fraction thus obtained was subjected to thin-layer chromatography (hexane–ethyl ether–formic acid 65:35:2 v/v) four major labeled fractions were observed: fatty acid methyl ester (R_F 0.73), ω-hydroxy acid methyl ester (R_F 0.35), an unknown (R_F 0.28), and 10,16-dihydroxy acid methyl ester (R_F 0.16). The R_F of the unknown is consistent with the acetal derivative of the methyl ester of 16-oxo-9- or 10-hydroxypalmitic acid. Acetylation of the labeled unknown gave a product less polar

than the unknown suggesting the occurrence of an alcohol function. Treatment of this material with dinitrophenylhydrazine and sulfuric acid followed by treatment of the product with 14% BF_3 in methanol to esterify any free acid generated during the acid treatment gave a single radioactive thin-layer chromatographic component (R_F 0.59 in ethyl ether–hexane–methanol–formic acid, 40:10:1:2 v/v). Treatment of the labeled hydrazone with pyruvic acid followed by reduction of the generated aldehyde with $NaBH_4$ gave a radioactive component of an R_F (0.48) identical with that of 10,16-dihydroxymethyl palmitate in the above solvent system. Acetylation followed by tlc showed that the radioactivity coincided with authentic 10,16-diacetoxymethyl palmitate. Furthermore, radio gas–liquid chromatography of the acetylated product showed that the radioactivity coincided with the mass of authentic 10,16-diacetoxymethyl palmitate (Figure 4). As shown in Scheme II these results strongly suggest that palmitic acid was incorporated into 16-oxo-9- or 10-hydroxypalmitic acid.

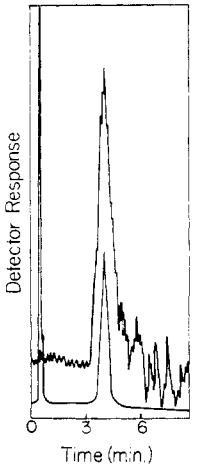
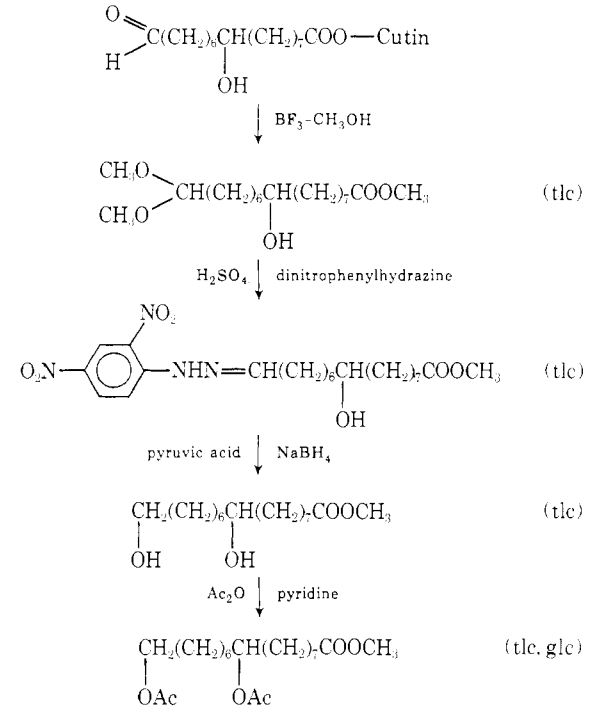


FIGURE 4: Radio gas–liquid chromatogram of 9- or 10,16-diacetoxymethyl palmitate derived by treatment of the dinitrophenylhydrazone with pyruvic acid followed by $NaBH_4$ and subsequent methylation and acetylation. Top tracing, radioactivity; bottom tracing, flame ionization detector response obtained with authentic 10,16-diacetoxymethyl palmitate. Glc was done with 5% OV-1 on 80–100 mesh Gas Chrom Q packed in 6 ft × 0.25 in. o.d. coiled stainless steel column held at 270°.

Identification of the Semicarbazone. A second approach used to show that labeled 16-oxo-9- or 10-hydroxypalmitic acid was generated from the exogenous [1-^{14}C]palmitic acid is outlined in Scheme III. Treatment of the insoluble material obtained from leaf slices, which were incubated with [1-^{14}C]-palmitic acid, with semicarbazide followed by treatment of the recovered residue with a partially purified preparation of cutinase (R. E. Purdy and P. E. Kolattukudy, 1973) gave four major thin-layer chromatographic components (Figure 5). Components I, II, and III were identified to be fatty acid, ω-hydroxy fatty acid, and 9- or 10,16-dihydroxypalmitic acid by their R_F values. Fractions II and III were also identified by radio gas–liquid chromatography of their acetylated methyl esters and their CrO_3 oxidation products (data not shown). Fraction IV, which was suspected to be the semicarbazone, when treated with pyruvic acid followed by reduction of the generated aldehyde with $NaBH_4$ gave a labeled material with an R_F value identical with that of 9- or 10,16-dihydroxypalmitic acid. Treatment of this material with BF_3–CH_3OH gave a labeled product which showed the same R_F as the 9- or 10,16-dihydroxymethyl palmitate. Acetylation of the methyl ester followed by tlc showed that the labeled product was identical with 9- or 10,16-diacetoxymethyl palmitate. This identification was further confirmed by radio gas–liquid chromatography which showed that the

SCHEME II



SCHEME III

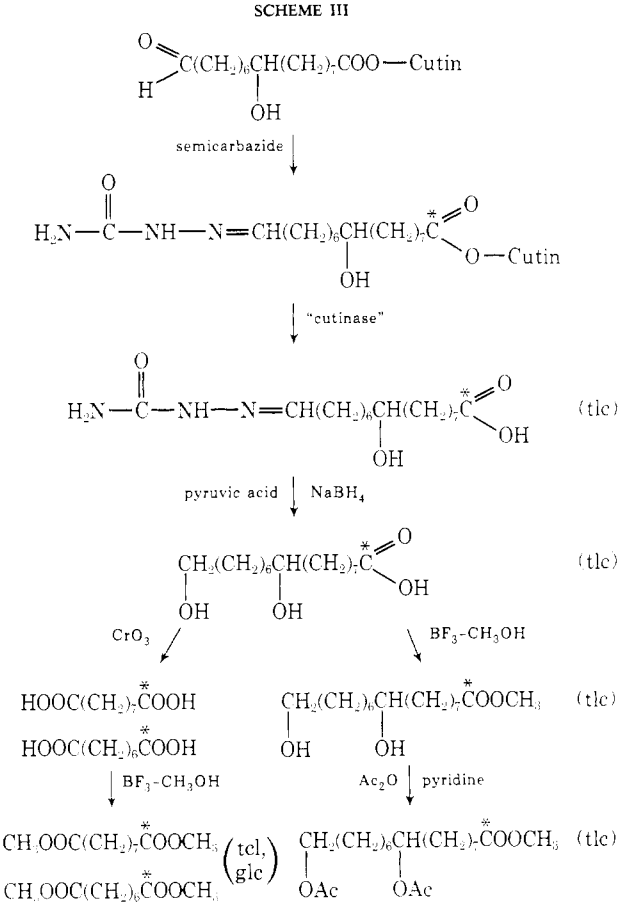


TABLE IV: Distribution of Radioactivity among the CrO_3 Oxidation Products of 10,16-Dihydroxypalmitic Acid and the 16-Oxo Compound Isolated *via* the Semicarbazone Derivative.[a]

	Radioactivity (%)	
Dicarboxylic Acid	16-Oxo Compound	10,16-Dihydroxy Compound
C_8	41.2	11.4
C_9	49.0	53.7
C_{10}	9.8	34.9

[a] 10,16-Dihydroxyhexadecanoic acid (from fraction III of Figure 5) and the dihydroxyhexadecanoic acid derived from the semicarbazone (fraction IV of Figure 5) of the 16-oxo-9- or 10-hydroxyhexadecanoic acid were oxidized with CrO_3 and the dicarboxylic acids were analyzed as dimethyl esters by glc as described under Table III.

label coincided with the mass of authentic material in a manner similar to that shown in Figure 4.

In order to determine the position of the in-chain hydroxyl function in the labeled oxo compound the labeled dihydroxy acid obtained from the procedure discussed above was degraded with CrO_3 and the resulting dicarboxylic acids were subjected to radio gas–liquid chromatography (Table IV). It is clear that C_8 and C_9 were the major labeled products followed by C_{10}. On the other hand, the 10,16-dihydroxypalmitic acid fraction released by cutinase from the same sample of cutin (fraction III, Figure 5) gave mainly C_9 and C_{10} dicarboxylic acids followed by C_8. Thus, the dihydroxypalmitic acid fraction contained mainly the 10,16-dihydroxy isomer while the 9-hydroxy isomer predominated in the 16-oxo compound fraction. These results are in agreement with the mass spectral data discussed in a previous section.

Identification of the Amino Derivative. In a third approach to confirm that 16-oxo-9- or 10-hydroxypalmitic acid was one of the major labeled components derived from exogenous [1-^{14}C]palmitic acid in very young *V. faba* leaves, the labeled cutin derived from this system was reduced with $LiAlH_4$ after treatment with hydroxylamine. The oxo compounds present in the original cutin should be converted by these treatments into amines. Therefore the amount of radioactivity in the basic fraction obtained from the hydrogenolysis step in itself indicates presence of labeled oxo compound in the polymer. Results of complete analysis of the basic and acidic fractions (Table V) showed that 24.2% of the radioactivity was contained in the basic fraction. All the radioactivity in the basic fraction was contained in a thin-layer component (chloroform–methanol–concentrated ammonia, 65:15:4) which showed an R_F identical with that of 16-aminohexadecane-1,9- or -10-diol which had been previously identified by mass spectrometry (Figure 6). N-Acetylation of the radioactive component gave rise to a compound with an R_F identical with the N-acetylated 16-aminohexadecane-1,9- or -10-diol. The labeled material purified by repeated tlc was also subjected to glc–ms as the Me_3Si derivative. Furthermore, CrO_3 oxidation of the basic fraction followed by radio gas–liquid chromatography of the resulting dicarboxylic acid (as dimethyl esters) showed that C_8 and C_9 were the major labeled dicarboxylic acids followed by C_{10} (the data were essentially identical with that in Table IV). These results confirmed that the oxo compound from which the amine was

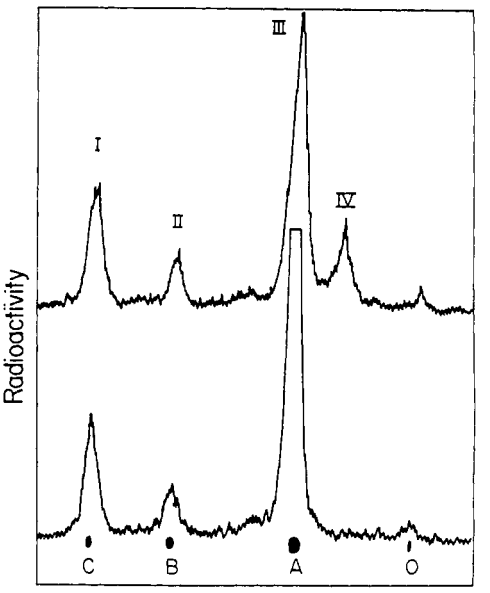


FIGURE 5: Radio thin-layer chromatograms of the lipid soluble products released by cutinase from semicarbazide treated cutin obtained from young (top) and old (bottom) leaves of *V. faba*, which were incubated with [1-^{14}C]palmitic acid. In each case 1 g of tissue was incubated with 424 nmol (25 μCi) of [1-^{14}C]palmitic acid for 4 hr at 30°. Tlc was done with ethyl ether–hexane–methanol–formic acid (40:10:1:2 v/v) as the solvent: O, origin; A, 10,16-dihydroxypalmitic acid; B, ω-hydroxypalmitic acid; C, palmitic acid.

derived was 16-oxo-9-hydroxypalmitic acid with smaller amounts of 16-oxo-10-hydroxypalmitic acid.

If the incorporation of [1-^{14}C]palmitic acid into the oxo compound identified by the preparation of the various derivatives is a reliable indication of the biosynthesis of the oxo compound, such incorporation measurements should agree with the observation that significant amounts of the oxo compound are synthesized only in very young tissue. Experimental results clearly showed that this was the case. For example, radio thin-layer chromatograms in Figure 5 show that the labeled semicarbazone fraction (fraction IV) was not detectable in the products derived from a fairly mature leaf whereas with very young leaves a substantial amount of label was found in this fraction. When the semicarbazide treated cutin preparation from young (4 mm) *V. faba* leaves which were incubated with [1-^{14}C]palmitic acid was depolymerized

TABLE V: Incorporation of [1-^{14}C]Palmitic Acid into Cutin Monomers in Young *V. faba* Leaves.[a]

	Radioactivity	
Fraction	cpm $\times 10^{-5}$	%
C_{16}-Alkanetriol	11.53	60.4
Alkanediol	1.83	9.6
Alkan-1-ol	1.12	5.9
Basic fraction	4.62	24.2

[a] [1-^{14}C]Palmitic acid (424 nmol, 25 μCi in 1.0 ml of water) was incubated with 2.4 g of tissue slices of young (2–4 mm) *V. faba* leaves for 4 hr at 30°. The crude cutin preparation was first treated with hydroxylamine and then with $LiAlH_4$ in tetrahydrofuran for 24 hr. The neutral products were separated into alkanol, alkanediol, and C_{16}-alkanetriol fractions by tlc in ethyl ether–hexane–methanol (8:2:1, v/v).

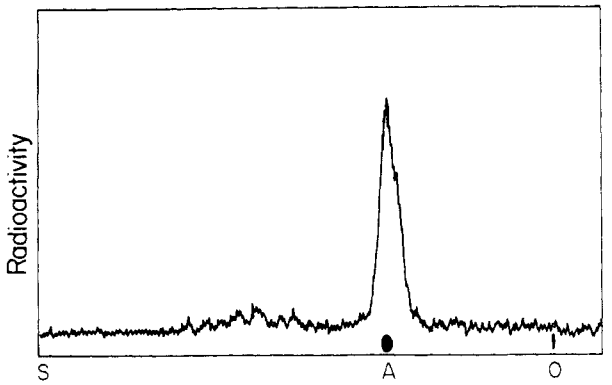


FIGURE 6: Radio thin-layer chromatogram of the basic fraction shown in Table V. Tlc was done with $CHCl_3$–CH_3OH–NH_4OH (65:14:4 v/v) as the solvent: O, origin; A, the major lipid component visible under uv light after spraying the plate with a 0.1% ethanolic solution of dichlorofluorescein.

with cutinase and the monomers released were reduced with $LiAlH_4$, 19.8% of the ^{14}C was found in the basic fraction while the corresponding value obtained with old (18–20 mm) leaves was only 0.39%. Similar results were also obtained with the hydroxylamine treatment procedures described earlier. Thus, the incorporation studies described above agree quite well with the observation that 16-oxo-9- or 10-hydroxypalmitic acid formation is a hallmark of young tissues. Incorporation of [1-^{14}C]palmitic acid into 16-oxo-9-hydroxypalmitic acid was also shown in embryonic shoots from germinating *V. faba* seeds by the procedures described for *V. faba* leaves. Since the experimental techniques were identical with those described above and the results were similar, the data are not presented here.

Incorporation of [G-^3H]-16-Hydroxypalmitic Acid into 16-Oxo-9- or 10-Hydroxypalmitic Acid. It has been shown that ω-hydroxypalmitic acid is converted into 10,16-dihydroxypalmitic acid in *V. faba* (Kolattukudy and Walton, 1972a; Walton and Kolattukudy, 1972b). Structural considerations suggest that ω-hydroxypalmitic acid should also serve as the precursor of the oxo compound. In order to test this possibility young *V. faba* leaves were incubated with [G-^3H]-ω-hydroxypalmitic acid and the insoluble crude preparation was hydrogenolyzed with $LiAlH_4$ after treating it with hydroxylamine. Over 5% of the total ^3H administered was incorporated into cutin and nearly one-fourth of it was in the basic fraction (Table VI). Thin-layer chromatographic analysis of the products showed that C_{16} triol and diol were the major products in the neutral fraction indicating direct conversion of the exogenous ω-hydroxy acid into the dihydroxy acid. Most of the label contained in the basic fraction was found to be in a component with an R_F identical with that of 16-aminohexadecane-1,9-diol which obviously was derived from the 16-oxo compound. If this incorporation does in fact reflect the natural synthesis of the 16-oxo compound, incorporation of exogenous 16-hydroxypalmitic acid into the oxo compound should reflect the age of the tissue as described earlier in this paper. That such was the case is shown by the data in Table VI which show that 23.2% of the label incorporated by the young tissue was in the oxo compound (isolated as the basic compound) while in the nearly mature leaves only 2.8% of the label was found in the oxo compound.

Mechanism of Formation of 16-Oxo-9- or 10-Hydroxypalmitic Acid. In-chain hydroxylation of 16-oxopalmitic acid or oxidation of 9- or 10,16-dihydroxypalmitic acid could result in the formation of the oxo compound. According to the latter mechanism, exogenous 9- or 10,16-dihydroxypalmitic acid might be expected to be directly converted into the oxo compound while such a conversion may not be expected from the former alternative. In order to test this possibility [G-^3H]-10,16-dihydroxypalmitic acid was incubated with young *V. faba* leaves and the insoluble material containing cutin was treated with hydroxylamine followed by hydrogenolysis of the recovered insoluble material. The basic fraction obtained from this procedure contained 14.6% of the total radioactivity incorporated into cutin (Table VI). Thin-layer chromatography of the basic material in chloroform–methanol–concentrated ammonia (65:35:4) showed that virtually all the radioactivity was contained in a component with an R_F identical with 16-amino-hexadecane-1,9-diol. Thin-layer chromatographic analysis of the neutral fraction showed that all the radioactivity was contained in hexadecane-1,7,16-triol. Therefore, it is concluded that the conversion of exogenous 10,16-dihydroxypalmitic acid into the corresponding 16-oxo compound is a direct oxidation rather than one which involved extensive degradation and resynthesis. Thus, specific oxidation of 9,16-dihydroxypalmitic acid appears to be the mechanism by which the oxo compound is formed in young *V. faba* leaves.

TABLE VI: Incorporation of [G-^3H]-16-Hydroxypalmitic Acid and [G-^3H]-10,16-Dihydroxypalmitic Acid into 16-Oxo-9- or 10-Hydroxypalmitic Acid in Young Leaves of *V. faba*.

Expt[a]	Substrate	Tissue	Radioactivity (dpm × 10^5) Administered	Triol	Diol	Oxo Compound (as amine)	Basic Fraction As of % Incorporation
1	[G-^3H]-16-Hydroxypalmitic acid	Young 3–5 mm (1.5 g)	54	1.36	0.61	0.594	23.2
		Old, 18–20 mm (1.5 g)	54	3.66	2.24	0.17	2.8
2	[G-^3H]-10,16-Dihydroxypalmitic acid	Young, 3–5 mm (2.9 g)	108	7.79		1.34	14.6
3	[G-^3H]-10,16-Dihydroxypalmitic acid	Young, 3–5 mm (1 g)	54	3.2		0.34	9.7
		Old, 18–20 mm (1 g)	54	5.8		0.26	4.3

[a] In all experiments tissue was incubated for 4 hr at 30°. The crude cutin was treated first with hydroxylamine and then with $LiAlH_4$. The neutral fraction and basic fraction were analyzed by tlc with ethyl ether–hexane–methanol (8:2:1 v/v) and chloroform–methanol–NH_4OH (65:15:4 v/v), respectively. Only the ^3H content of the tlc fraction which had the same R_F as 16-amino-hexadecane-1,9-diol is included under the 16-oxo compound column.

It is possible that the activity of the ω-hydroxy acid dehydrogenase involved in the formation of the 16-oxo compound decreases with age. If such is the case, the oxidation of exogenous labeled 10,16-dihydroxy acid to the aldehyde should be more efficient in the young leaves than in the more mature leaves. On the basis of the total radioactivity incorporated into cutin the young tissue converted exogenous 10,16-dihydroxypalmitic acid into the aldehyde more than twice as efficiently as the nearly mature tissue. It was observed that conversion of exogenous palmitic acid and ω-hydroxypalmitic acid into the aldehyde more closely reflected the correlation of the aldehyde content of the tissue with the physiological state (Table II) than the oxidation of exogenous 10,16-dihydroxypalmitic acid. Therefore, the regulation of the formation of the 16-oxo compound of the polymer may involve something more than the activity of the appropriate dehydrogenase.

The occurrence of 16-oxo-9- or 10-hydroxypalmitic acid in the cuticular polymer and the decrease in its proportion during tissue expansion suggests that the oxo compound may play an important role in the cuticular structure. It is possible that such a functional group is involved in the anchoring of the polymer to the tissue by means of acetal or Schiff base type linkages. Results previously published from this laboratory indicated that in cutin, which contains substantial amounts of epoxy and polyhydroxy C_{18} acids, *cis*-1,4-pentadiene containing acids might be the precursors of the ether-bonded portion of the polymer (Kolattukudy *et al.*, 1973). In cutin which contains very little dienoic acids, the oxo compound discussed in this paper might be involved in forming acetal type cross-linkages. Direct evidence for such a hypothesis is being sought at the present time in this laboratory and preliminary results indicate that such linkages do occur in *V. faba* cutin. Furthermore, hydroxylamine treatment of cutin followed by hydrogenolysis and product analysis by a combination of gas–liquid chromatography and mass spectrometry showed that 16-oxo-9- or 10-hydroxypalmitic acid occurs in other plants also (V. P. Agrawal and P. E. Kolattukudy, unpublished observations). Thus this compound is probably of widespread occurrence and may have significant functions in the cuticular structure of plants. More recent measurements of the absolute amounts of the 9-hydroxy and 10-hydroxy isomers showed that as the leaves developed the formation of the 9-hydroxy isomer leveled off while the synthesis of the 10-hydroxy isomer was increasing rapidly. Thus it appears that either the enzyme system responsible for the formation of the 9-hydroxy aldehyde is shut off as the leaf matures, or its specificity changes with the age of the leaf.

Acknowledgments

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